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Award Number: DAMD17-00-1-0090

TITLE: COX-2 and Prostate Cancer Angiogenesis

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REPORT DATE: March 2002

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
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20020913 078

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE March 2002	3. REPORT TYPE AND DATES COVERED Annual (1 Mar 01 - 28 Feb 02)

4. TITLE AND SUBTITLE COX-2 and Prostate Cancer Angiogenesis	5. FUNDING NUMBERS DAMD17-00-1-0090

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9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES
Reports contains color

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 Words)

Cyclooxygenase-2 (COX-2) is an inducible enzyme which catalyzes the conversion of arachidonic acid to prostaglandins and has previously been demonstrated to play a role in carcinogenesis. We demonstrated that COX-2 and one of its major prostaglandin products, PGE2, are mediators of hypoxia-induced increases in a potent angiogenic factor, VEGF, in a human prostate cancer cell line. In these studies we are determining (1) The optimal dosing and timing of administration of a COX-2 inhibitor (NS-398) in an animal model of prostate cancer (2) and (3) the mechanisms underlying the observed effects of COX-2 and PGE2 on hypoixa-induced upregulation of VEGF and tumor angiogenesis. Over the past year, we have extended our in vivo studies to determine the optimal timing of administration of NS-398 in the *in vivo* model system. We demonstrated that PGE2 induces the protein expression of a central regulator of hypoxic effects, hypoxia-inducible factor-1α (HIF-1α) and induces its nuclear localization. Our data indicates that NS-398 blocks hypoxic effects on HIF-1α protein while PGE2 restores hypoxic effects, even in the presence of NS-398. Finally, we demonstrated that several kinase pathways, most notably the MAP kinase pathway, are involved in PGE2 effects on HIF-1α protein stabilization.

14. SUBJECT TERMS Angiogenesis, VEGF, COX-2	15. NUMBER OF PAGES 17
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT Unclassified	18. SECURITY CLASSIFICATION OF THIS PAGE Unclassified	19. SECURITY CLASSIFICATION OF ABSTRACT Unclassified	20. LIMITATION OF ABSTRACT Unlimited

NSN 7540-01-280-5500

Standard Form 298 (Rev. 2-89)
Prescribed by ANSI Std. Z39-18
298-102

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INTRODUCTION

Cyclooxygenase-2 (COX-2) is an inducible enzyme which catalyzes the conversion of arachidonic acid to prostaglandins. COX-2 plays a key role in cancer (1-4) and COX-2 inhibition prevents and treats colon cancer (5-7). Both COX-2 and its major derived prostaglandin product (PGE_2) have been implicated as stimulators of tumor angiogenesis (8-13).

We previously demonstrated that COX-2 expression is increased in human prostate cancer tissues (14) and that a COX-2 inhibitor selectively induces apoptosis in a prostate cancer cell line (15). We also demonstrated that treatment of human prostate tumor-bearing mice with a selective COX-2 inhibitor (NS-398) significantly reduces tumor size, microvessel density and levels of a potent tumor angiogenic factor, vascular endothelial growth factor (VEGF) (16).

Our *in vitro* studies with a highly invasive human prostate cancer cell line, PC-3ML, demonstrated that cobalt-chloride simulated hypoxia induced VEGF upregulation. In that same report, VEGF upregulation by cobalt chloride simulated hypoxia was prevented by NS-398 treatment and restored by the addition of PGE_2 (one of the major prostaglandin products of the reaction catalyzed by COX-2) (17).

Based on our preliminary data, we hypothesized that COX-2 and the resultant PGE_2 are mediators of hypoxia-induced effects on VEGF in prostate cancer cells. We further hypothesized that PGE_2 mediates hypoxic upregulation of VEGF by modulating hypoxia-inducible factor-1α (HIF-1α), the major transcriptional regulator of VEGF expression. The first task of our initial proposal was to determine the optimal dosing and timing of administration of NS-398 (COX-2 inhibitor) in prostate-tumor bearing mice. The second task was to determine the mechanisms underlying the observed effects of the COX-2 inhibitor and PGE_2 on hypoxia-induced upregulation of VEGF. Specifically, we originally proposed to determine whether PGE_2 itself regulates HIF-1α and VEGF expression levels. Finally, in Task 3, studies were proposed to determine whether PGE_2 modulates VEGF transcription by enhancing the binding of HIF to the promoter region of the VEGF gene.

In last year's annual report, we demonstrated that NS398 induced a dose-dependent inhibition of prostate tumor growth in nude mice. Of the three doses tested, maximal tumor growth inhibition was observed with the highest dose (3.0 mg/kg/body weight of NS398 administered three times weekly, intraperitonally, for a total of 14 weeks). There was no observable toxicity to the animals at any of the doses tested. Over the past year, we have begun studies testing whether the timing of administration of NS398 effects tumor take rates or growth curves.

In last year's report, we demonstrated that true hypoxia increases VEGF secretion by PC-3ML human prostate cancer cells and that a COX-2 inhibitor (NS398) blocks this hypoxic effect, while co-administration of one of the COX-2 derived prostaglandin products (PGE_2) restores hypoxic effects on VEGF, even in the presence of NS-398. We further demonstrated that PGE_2 did not modulate HIF-1α mRNA transcription but, rather, modulated HIF-1α expression at the protein level. Over the past year we have studied the interactive effects of hypoxia, COX-2 and PGE_2 on HIF-1α protein expression and nuclear localization. In addition, we investigated the possible kinase pathways that may be involved in nuclear localization and activation of HIF-1α induced by both hypoxia and PGE_2.

BODY

Task 1. Perform studies using a mouse model of human prostate cancer to determine the in vivo efficacy of a selective COX-2 inhibitor as an anti-tumor and anti-angiogenic agent.

Last year, we reported on the results of Task 1A (Dose studies with NS-398) and demonstrated that the highest dose studied (3 mg/kg/body weight given three times weekly, intraperitoneally) caused maximal tumor growth inhibition. In those studies, the NS-398 was administered 2 weeks after s.c. inoculation of PC-3 ML human prostate cancer cells into nude mice. The treatment was begun at that time because that is when tumors were visible and palpable in all animals. Over the past year we have preliminary data investigating whether administration of the COX-2 inhibitor prior to tumor inoculation decreases tumor take rates or slows their growth in nude mice.

1B. Optimal Timing of Drug Therapy

12 nude mice were randomized into two groups to receive either (1) Vehicle (as control) or (2) NS-398, 3 mg/kg/body weight intraperitoneally three times weekly beginning two weeks prior to s.c. inoculation of 1 million PC-3 ML human prostate cancer cells. We have data on tumor take rates and growth curves 7 weeks after inoculation of tumor cells (the experiment is still ongoing). Fig. 1 demonstrates that NS398 treatment given two weeks prior to tumor cell inoculation does not modulate tumor take rates (tumor take rate was 100% in both groups of animals). However, the preliminary data indicates that NS398 treated animals do have smaller tumors initially as compared to control animals and slower tumor growth rates throughout the 7 weeks of treatment (Fig. 1, below).
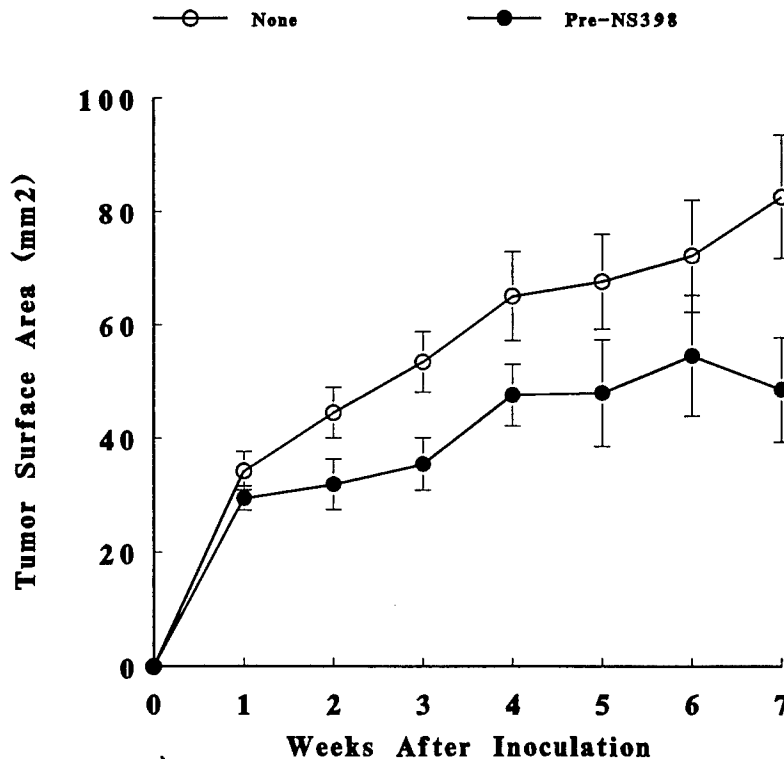

Fig. 1. Effect of NS398 pre-treatment on PC-3 cell tumor growth in nude mice. Mice were randomly divided in into two groups with 8 mice in each. The first group of mice received vehicle (PBS) only as control. The second group of mice received NS398 (3mg/kg body weight, 3 times/week, i.p.) 2 weeks prior to tumor cell inoculation. The treatment was continued in all experimental period. Data were expressed as means ± SE.

*Task 2:* **Determine the interactive effects of hypoxia, COX-2 and PGE₂ on HIF-1α protein expression and nuclear localization.**

Hypoxia induces HIF-1α protein stabilization, particularly in the nuclear fraction. HIF-1α protein is a cytosolic protein which, upon stimulation by various factors, undergoes nuclear translocation. We examined the effect of hypoxia on the expression and subcellular localization of HIF-1α protein. Western blot analysis of protein samples extracted either from the cytosol or the nucleus revealed that PC-3 ML cells expressed a low level of HIF-1α protein in the cytosol, but a relatively high level in the nucleus under normoxic conditions. Hypoxia (12 h) did not have a significant effect on cytosolic protein levels but induced a band shift from low molecular weight to high molecular weight, suggesting increased cytosolic HIF-1α protein phosphorylation after hypoxia. Hypoxia induced a significant increased in HIF-1α protein levels in the nuclear fraction, suggesting that hypoxia induced HIF-1α protein translocation from the cytosol to the nucleus (Fig. 2). PGE₂ upregulates HIF-1α protein expression, with a peak induction noted at 4-8 h after treatment.

PGE₂ induces HIF-1α protein in the cytosol and the nucleus under both normoxic and hypoxic conditions. We next examined the effect of PGE₂ on HIF-1α protein expression. Fig. 2 demonstrates that PGE₂ upregulated HIF-1α levels in both the cytosolic and nuclear fractions in normoxic cells. The PGE₂-induced HIF-1α protein expression in the cytosol was first noted 2 h after treatment which was prior to the induction observed in the nuclear fraction (4 h). The combination of PGE₂ and hypoxia resulted in greater increases in HIF-1α protein levels, particularly in the nuclear fraction, than those seen with either treatment alone. Of note, the combination of hypoxia and 1 μM PGE₂ induced a band shift in the cytosolic fraction, which, again, may represent an increase in HIF-1α protein phosphorylation (Fig. 2). These data indicate that PGE₂ and hypoxia act both independently and synergistically to increase HIF-1α protein levels and nuclear localization in PC-3 ML human prostate cancer cells.
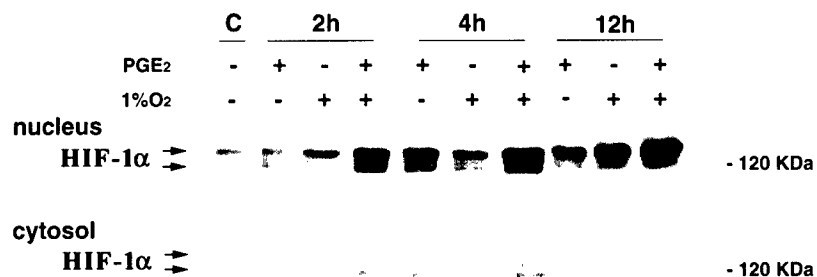
**Fig. 2. *PGE₂-induced HIF-1α protein stabilization.*** PC-3ML cells were cultured in serum-free medium and treated with either 1 μM PGE₂, or 1% O₂, or combination of PGE₂ (1 μM) and 1% O₂ for various times as indicated. Proteins in the nuclear and cytosolic fractions were isolated and subjected to Western blot analysis. Twenty μg of protein were loaded in each lane.

PGE₂ induced nuclear localization of HIF-1α protein. Previous studies have determined that endogenous HIF-1α translocates to the nucleus in hypoxic cells and that this translocation is essential for its transactivation and, possibly, for its stabilization. Our data from Western blotting indicated that both hypoxia and PGE₂ significantly increased HIF-1α protein levels, particularly in the nuclear fraction. We, therefore, investigated the possibility that PGE₂ promotes HIF-1α protein nuclear translocation independent of hypoxia using a nucleo-cytosolic trafficking assay. We generated a pEGFP-HIF-1α vector which expresses GFP-tagged HIF-1α protein. The vector was transiently transfected into PC-3ML cells. The cells were then treated with 1 μM PGE₂ for various time periods and the intracellular localization of the HIF-1α determined by confocal laser microscopy.
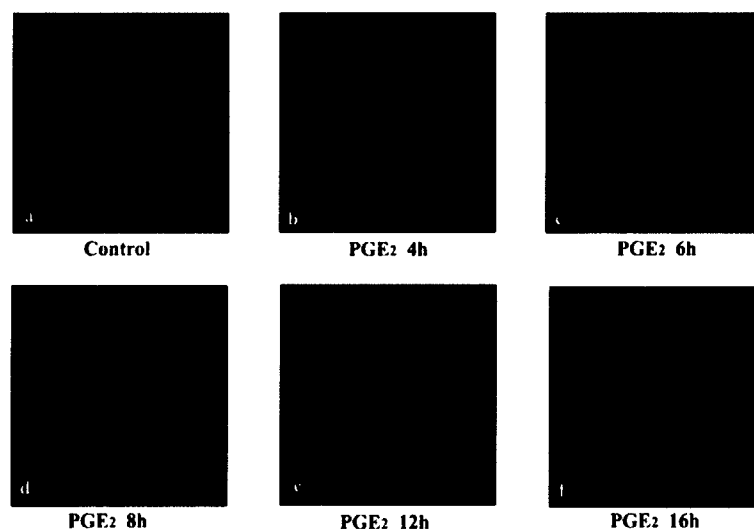
Fig. 3. PGE₂-induced nuclear translocation of HIF-1α protein. PC-3ML cells were transiently transfected with the chimeric pEGFP-HIF-1α construct. After transfection, cells were cultured under normoxic conditions and treated with either vehicle as control (*A*) or 1 μM PGE₂ for 4 h (*B*), 6 h (*C*), 8 h (*D*), 12 h (*E*), and 16 h (*F*). Cells were then fixed with 4% formaldehyde in PBS and covered by mounting medium. The intensity and subcellular localization of pGFP-HIF-1α were examined using a laser scanning confocal microscope.

The Roles of MAPK/Erk1/2 and PI-3K/AKT in PGE₂-induced HIF-1α Expression .

We next determined the possible involvement of several intracellular kinase pathways in PGE₂-induced HIF-1α expression. As shown in Fig. 4A, Western blot analysis demonstrated that PD98059, a MAPK inhibitor (50 μM), had no effect on basal HIF-1α expression. However, PD98059 significantly suppressed PGE₂-induced upregulation of HIF-1α protein expression. The effect of PD98059 was observed in both the nucleus and cytosol and was dose-dependent (Fig. 4B). LY294002, a PI-3K inhibitor, strongly inhibited basal HIF-1α nuclear accumulation but only partially inhibited PGE₂-inducible nuclear and cytosolic HIF-1α protein levels. Staurosporine, a protein kinase C inhibitor, had no effect on either basal and PGE₂-induced HIF-1α expression in both the nuclear and cytosolic fractions (Fig. 4A). These results demonstrate that the MAP kinase inhibitor (PD98059) specifically inhibits PGE₂-induced stabilization and nuclear localization of HIF-1α in this cell line, whereas the PI-3K inhibitor (LY294002) modulates both the basal and PGE₂-induced effects on the protein.
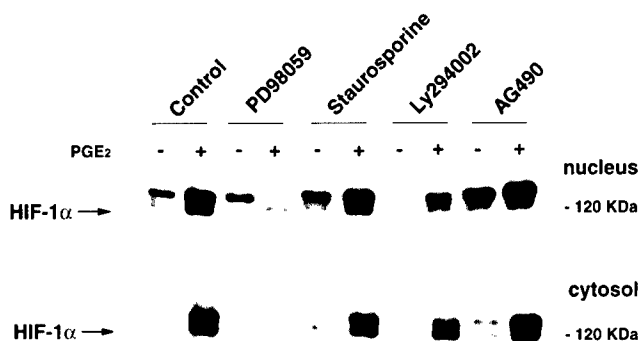
Fig. 4. The effects of kinase inhibitors on PGE₂-induced HIF-1α stabilization. *A*, PC-3ML cells were pre-treated with 50 μM PD98059, or 5 μM LY294007, or 20 nM staurosporine for 20 h, incubations were continued with or without 1 μM PGE₂ for an additional 4 h. Proteins in the nuclear and cytosolic fractions were then isolated and subjected to Western blot analysis. *B*, cells were pre-treated with various concentrations of PD98059, as indicated for 20 h prior to 1 μM PGE₂ treatment for 4 h. Proteins in the nuclear fraction were then isolated and subjected to Western blot analysis.

Inhibition of COX-2 Activity Suppressed Hypoxia-induced HIF-1α Nuclear Expression. We previously reported that NS398, a selective COX-2 inhibitor, can prevent VEGF upregulation in response to cobalt-chloride induced hypoxia (32). Similar results have been observed when the same cell line was exposed to true hypoxia (1% O_2). We tested whether this effect of NS398 is due to inhibition of hypoxic effects on HIF-1α protein expression. As shown in Fig. 5, Western blotting confirmed that both PGE_2 and hypoxia significantly upregulated HIF-1α protein levels, particularly the higher molecular weight band of the protein. Pretreatment of cells with a selective COX-2 inhibitor, NS398 (50 μM), for 2 d inhibited both the basal and hypoxia-induced nuclear accumulation of the protein. We next sought to determine whether this inhibition could be overcome by exogenous PGE_2. Fig. 5 demonstrates that the addition of PGE_2 completely restored the inhibitory effects of NS398 on both basal and hypoxia-induced upregulation of HIF-1α protein. These results indicate that PGE_2 production via the COX-2-catalyzed pathway plays a critical role in both basal and hypoxia-stimulated regulation of HIF-1α protein.
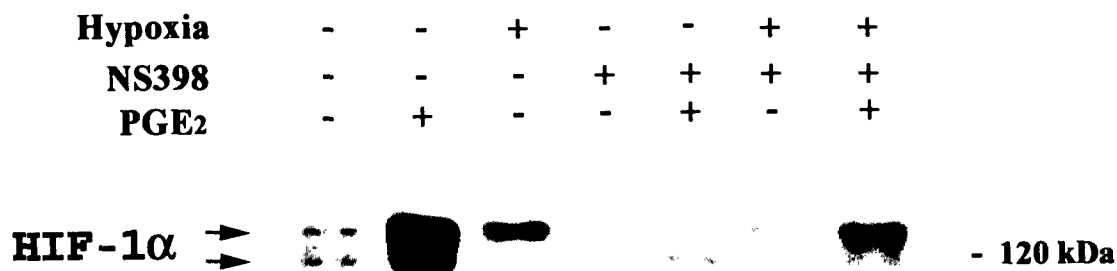
Fig. 5. NS398 inhibits hypoxia-induced nuclear accumulation of HIF-1α. PC-3ML cells were cultured in serum-free medium for 24 h prior to various treatments as following: lane 1, vehicle only as control; lane 2, 1 μM PGE_2 for 4 h; lane 3, 1% O_2 for 12 h; lane 4, 50 μM NS398 for 48 h; lane 5, 50 μM NS398 and 10 μM PGE_2 for 48 h; lane 6, 50 μM NS398 for 36 h followed by 1% O_2 for 12 h; lane 7, 50 μM NS398 plus 10 μM PGE_2 for 36 h prior to 1% O_2 treatment for 12 h. Proteins in the nuclear fraction were then isolated and subjected to Western blot analysis.

KEY RESEARCH ACCOMPLISHMENTS

Over the past (2nd year of funding) we have:

● Obtained preliminary data on the effects of a COX-2 inhibitor on tumor take rates and tumor growth rates when given prior to cancer cell inoculation in a nude mouse model. The preliminary results do not indicate that COX-2 inhibitors prevent tumor take when given two weeks prior to tumor cell inoculation. In future studies, we will try to pre-treat for a longer time period prior to tumor cell inoculation.

● Demonstrated that PGE_2 increases HIF-1α protein levels in PC-3 ML prostate cancer cells.

● Demonstrated that PGE_2 induces nuclear localization of HIF-1α protein in PC-3 ML cells.

● Elucidated the kinase pathways involved in the observed PGE_2 effects on HIF-1α protein.

● Demonstrated that a COX-2 inhibitor specifically blocks the induction of HIF-1α protein by hypoxia and that PGE_2 can restore this effect of hypoxia.

REPORTABLE OUTCOMES

Publications

We recently submitted a manuscript to the **Journal of Biological Chemistry** which arose from these studies: **Liu X.H., Kirschenbaum A., Lu M., Yao S., Dosoretz A., Holland J.F. and Levine A.C. Prostaglandin E_2 induces hypoxia-inducible factor-1α stabilization and nuclear localization in a human prostate cancer cell line.** We received a response from the JBC on 2/27/02 (manuscript #01095) which indicated that the manuscript would be considered for publication after revision and we are in the process of revising it according to the reviewers' critiques.

In addition, the P.I. and Co-Investigators wrote an invited review which includes some of the findings derived from these studies. The DOD funding is credited in the article

Kirschenbaum A., Liu X-H., Yao S., and Levine A.C.: The role of cyclooxygenase-2 in prostate cancer. Urology (Suppl 2A): 127-131, 2001. (Reference 18, Appendix).

Presentations

Dr. Alice Levine (P.I.) was an invited speaker at two National Meetings and reported some of the findings of the DOD-funded studies in her talks.

"The Role of Cox-2 in Prostate Cancer", Alzheimer's Disease, Cancer and the Search for a Better Aspirin, New York, NY June 2001.

"Cox-2 Inhibitors as Anti-Angiogenic Therapy", Fourth International Symposium on Anti-Angiogenic Agents, Dallas, Texas, January 2002.

Funding Applied For Based On Work Supported by This Award

NIH/NCI RO1, P.I. Alice C. Levine, M.D., "COX-mediates hypoxic stabilization of HIF-1 alpha", initial score 26th percentile, first revision submitted March 1, 2002 to NIH.

CONCLUSIONS

Task 1: In Vivo Studies with NS-398 (COX-2 Inhibitor)

We have demonstrated that a selective COX-2 inhibitor, NS-398, is effective anti-tumor therapy in a nude mouse model of human prostate cancer. The highest dose utilized (3mg/kg/body weight given intraperitoneally three times weekly for 15 weeks) dramatically slowed tumor growth without any evidence of toxicity in the animals. Over the past year, we have initiated studies to determine whether administering the COX-2 inhibitor prior to tumor cell inoculation will decrease tumor take rates. Thus far, at least when given only two weeks prior to tumor cell inoculation, the COX-2 inhibitor did not appear to prevent tumor take. We will expand these studies over the coming year and administer the COX-2 inhibitor earlier to determine if this renders it a more efficacious means of preventing tumor take.

Task 2: Determine the Interactive Effects of Hypoxia, COX-2 and PGE$_2$ on HIF-1α Protein Expression and Nuclear localization

We modified Task 2 last year, based upon our initial findings which indicated that PGE$_2$ did not have any effect on HIF-1α mRNA levels, but, rather, increased its protein levels. We therefore studied the regulation of HIF-1α protein levels and nuclear localization. As HIF-1α is a nuclear transcriptional factor (involved in the transcriptional activation of a variety of hypoxia-induced genes), its nuclear localization is a key step in its activation.

Over the past year, we have demonstrated that PGE$_2$ increases HIF-1α protein levels and induces its nuclear localization in PC-3 ML human prostate cancer cells. In addition, we demonstrated that several kinase pathways, most notably the MAP kinase pathway, mediate this effect. Finally, we demonstrated that COX-2 and PGE$_2$ mediate hypoxic effects on HIF-1α protein and that a selective COX-2 inhibitor can prevent hypoxic upregulation of HIF-1α protein in human prostate cancer cells.

These observations have important clinical applications in prostate cancer therapy. Intratumoral hypoxia cannot be controlled. Most hypoxic signaling occurs via HIF. We have demonstrated that a selective COX-2 inhibitor can prevent the upregulation of this central hypoxic signaling factor. Therefore, at least in this human prostate cancer cell line, COX-2 inhibition may prevent hypoxic upregulation of a variety of HIF-controlled genes including VEGF and glycolytic enzymes and thereby inhibit tumor growth and angiogenesis.

Task 3: Examine the intracellular interactions between PGE$_2$, HIF-1 and VEGF in PC-3 ML prostate cancer cells.

As originally proposed, the studies outlined in Task 3 will be completed in the coming (final) year of these studies. We will determine whether, in addition to the demonstrated effects on HIF-1 protein levels and nuclear localization, COX-2 and one of its resultant prostaglandin products (PGE$_2$), also modulate the binding of HIF-1 to the VEGF promoter and its transcriptional activity.

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THE ROLE OF CYCLOOXYGENASE-2 IN PROSTATE CANCER

ALEXANDER KIRSCHENBAUM, XIN-HUA LIU, SHEN YAO, AND ALICE C. LEVINE

ABSTRACT

Cyclooxygenase-2 (COX-2) is the inducible isozyme of COX, a key enzyme in the conversion of arachidonic acid to prostaglandins and other eicosanoids. COX-2 is highly expressed in a number of human cancers and cancer cell lines, including prostate cancer. We studied the immunohistochemical expression of COX-2 in the human prostate gland. The enzyme is strongly expressed in smooth muscle cells of both the normal and cancerous prostate. Its expression in noncancerous epithelial cells is limited to the basal cell layer. In prostatic inflammation, luminal epithelial cells surrounded by lymphocytes are induced to express the enzyme. COX-2 is expressed in the epithelial cells of high-grade prostatic intraepithelial neoplasia and cancer. We have demonstrated that treatment of human prostate-cancer cell lines with a selective COX-2 inhibitor induces apoptosis both in vitro and in vivo. The in vivo results also indicate that the COX-2 inhibitor decreases tumor microvessel density and angiogenesis. COX-2 inhibitors can prevent the hypoxic upregulation of a potent angiogenic factor, vascular endothelial growth factor. These results indicate that COX-2 inhibitors may, therefore, serve as effective chemopreventive and therapeutic agents in cancer of the prostate. UROLOGY 58 (Suppl 2A): 127–131, 2001. © 2001, Elsevier Science Inc.

Cyclooxygenase (COX), also referred to as prostaglandin (PG) endoperoxide synthase, is a key enzyme in the conversion of arachidonic acid to PGs and other eicosanoids. Two isoforms of COX have been identified. COX-1 is expressed constitutively in many tissues and cell types, whereas COX-2 is inducible by a variety of factors, including cytokines, growth factors, and tumor promoters.[1] COX-2 is highly expressed in a number of human cancers and cancer cell lines, including prostate cancer.[2,3] Although selective inhibitors of COX-2 activity appear to be effective in colon-cancer prevention and treatment,[4–6] little attention has previously been given to COX-2 and its relation to prostate cancer.

CYCLOOXYGENASE-2 AND CANCER: POSSIBLE MECHANISTIC LINKS

The initial interest in COX-2 grew out of the observation that patients taking nonsteroidal anti-inflammatory drugs (NSAIDs), which inhibit the activity of both COX-1 and COX-2, had a decreased risk of colon cancer.[7] The COX enzymes catalyze the 2-step conversion of arachidonic acid to PGH_2. The first step is the cyclooxygenase step in which oxygen is incorporated to form the intermediary PGG_2. The second step is the peroxidation step in which PGG_2 is reduced to form PGH_2, which can then be converted to a number of PGs. During these 2 reactions, free radicals are generated and xenobiotics can be oxidized to form mutagens. In addition, PGH_2, instead of being converted into other PGs, can be broken down to form a direct mutagen, malondialdehyde.[8,9] Inhibition of COX enzymes leads to an increase in arachidonic acid, which can stimulate the conversion of sphingomyelin to ceramide, a mediator of apoptosis (Figure 1).[10]

In colon cancer, much of the focus has been on the COX-2 isozyme, because, unlike the COX-1 isozyme, which is constitutively expressed in colonic epithelial cells, COX-2 is highly expressed only in cancerous epithelium.[8] Overexpression and persistent presence of COX-2 has been linked to promotion of tumorigenesis, resistance to apoptosis, and abnormal cell-cycle regulation.[11] It is well established that mutations in the adenomatous polyposis coli (APC) tumor-suppressor gene in patients with familial adenomatous polyposis
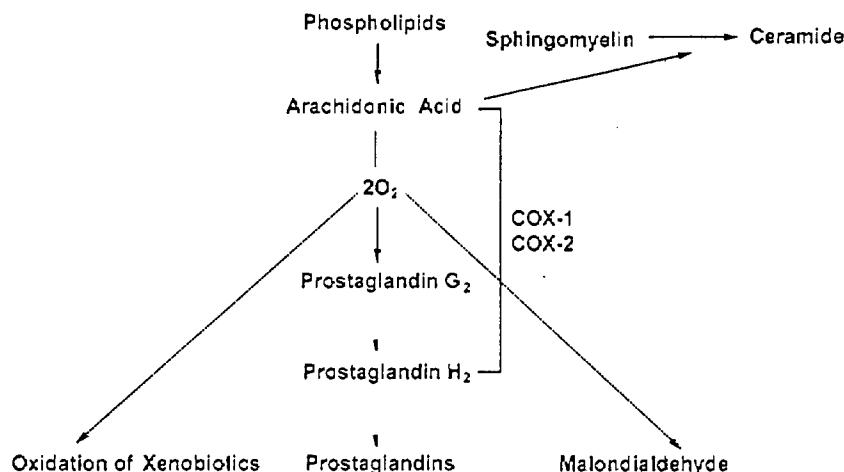
FIGURE 1. *Cyclooxygenase-1 and cyclooxygenase-2 (COX-1 and COX-2) catalytic pathways.*

predispose to the development of colon cancer. Mice carrying mutations in both the APC and COX-2 genes have greatly reduced numbers of polyps as compared with those carrying only the APC mutation.[12] These data suggest that the induction of COX-2 is an early event in adenoma formation in this model.

COX-2 has also been implicated at later stages of tumor progression. COX-2 overexpression has been shown to correlate with a more aggressive metastatic phenotype of human cancer cells.[13,14] Recent evidence provides a strong link between COX-2 expression and hypoxia-induced tumor angiogenesis.[15,16] PGE_2, a major COX-2–derived product, has been reported to stimulate angiogenesis.[17] Although it was initially believed that tumor cell–derived COX-2 was the culprit, recent reports have demonstrated that both endothelial- and stromal-derived COX-2 play a critical role in the regulation of tumor growth and angiogenesis. Using a corneal model, it was demonstrated that endothelial cell COX-2 is essential for angiogenesis.[18,19] In vivo experiments with Lewis lung carcinoma cells showed that tumor growth was markedly attenuated in COX-2-/-mice, with a decrease in tumor microvessel density. It was apparent that stromal-derived host COX-2 was critical for tumor growth, as the tumor cells did express COX-2, yet were unable to grow in the COX-2-/-host. In vitro studies with the COX-2-/-fibroblasts demonstrated that these cells were unable to secrete a key angiogenic factor, vascular endothelial growth factor (VEGF).[20]

PROSTAGLANDINS, NONSTEROIDAL ANTI-INFLAMMATORY DRUGS, AND PROSTATE CANCER

There is considerable evidence that the PG content of prostate-cancer cell lines and tissues is in-

creased.[21–24] Increased levels of PGs have also been reported in animal models of prostate cancer.[25,26] There is speculation that PGs may be involved in the carcinogenic effects of fats and hormones on the prostate.[27]

Despite the evidence that PG levels are elevated in malignant prostatic tissue, the results of epidemiologic studies determining the effects of NSAIDs on prostate cancer incidence have been inconclusive.[27] Although 1 such study demonstrated a trend toward decreased risk in NSAID users,[28] another report did not find a protective effect against the development of prostate cancer.[29]

PROSTATIC INFLAMMATION AND CANCER DEVELOPMENT

Proliferation associated with long-standing chronic inflammation has been implicated in the development of cancer in a variety of tissues including skin, urinary bladder, gastric mucosa, liver, and large bowel.[30–32] The human prostate gland is a common site of inflammation. It has been demonstrated that focal prostatic atrophy, which is associated with chronic inflammation, is proliferative.[33] An immunohistochemical study of focal prostatic atrophy confirmed that these lesions are highly proliferative and have a low apoptotic rate. These changes are due, at least in part, to overexpression of the antideath gene Bcl-2 and decreased expression of the cell-cycle regulatory gene p27.[34]

Immunohistochemical staining of human prostate tissues for COX-2 expression demonstrated that, in the noncancerous prostate, enzyme expression was limited to smooth muscle cells and some basal epithelial cells. Luminal cells of the noncancerous prostate were negative for COX-2 expression.[3] We noted, however, that luminal cells in areas of inflammation in noncancerous prostate

did demonstrate enzyme expression. These luminal cells were surrounded primarily by T lymphocytes. The same luminal cells that were induced by inflammation to express COX-2 also had increased expression of the antideath protein Bcl-2 and decreased expression of the cell-cycle regulatory protein p27 (unpublished data).

PROSTATIC INTRAEPITHELIAL NEOPLASIA

High-grade prostatic intraepithelial neoplasia (PIN) is the most likely precursor of prostatic cancer.[35] Increased expression of COX-2 protein was demonstrated in most PIN cells (86%) and their surrounding basal cells (75%).[3] A human PIN cell line was established by human papilloma virus–18 immortalization of PIN cell areas of radical-prostatectomy specimens. These cells were demonstrated by Western blotting to express prostate-specific antigen and cytokeratin 34βE12, thereby confirming their probable PIN cell origin.[36] We performed experiments with this cell line to determine the effects of inflammatory cytokines on COX-2 expression and PGE_2 secretion. Both tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) induced COX-2 expression (messenger RNA [mRNA] and protein) and PGE_2 secretion in a time- and dose-dependent fashion in the PIN cell line. IL-6–neutralizing antibodies prevented the upregulation of COX-2 expression induced by both IL-6 and TNF-α (unpublished data). These data demonstrate that inflammatory cytokines can increase COX-2 expression in PIN cells. As forced COX-2 expression in colon cells results in tumor development and progression,[11] these data provide a possible rationale for the use of COX-2 inhibitors in the prevention of the development and early progression of prostate cancer.

PROSTATE CANCER

Increased COX-2 expression has been described in a variety of human cancers.[37–42] Our immunohistochemical studies revealed that COX-2 staining in prostate cancer cells was intense and uniform, with 87% of samples demonstrating immunoreactivity. No significant relation was noted between Gleason score and staining intensity for COX-2 in a total of 62 regions (derived from 31 radical-prostatectomy specimens) examined.[3]

In vitro studies using the androgen-sensitive LNCaP cell line and the androgen-insensitive PC-3 cell line demonstrate that both cell lines express detectable amounts of COX-2 mRNA and protein and secrete PGE_2. We treated both cell lines with a selective COX-2 inhibitor, N-[2-(cyclohexyloxy)-4-nitrophenyl]-methanesulfonamide (NS-398), and demonstrated an induction of apoptosis.[43,44] In the LNCaP cells, the effect on apoptosis was preceded by phosphorylation and inactivation of Bcl-2.[43] Another group reported on the treatment of LNCaP and PC-3 cells with a different selective COX-2 inhibitor, celecoxib, and also demonstrated an induction of apoptosis.[44] Interestingly, they found that this effect was independent of Bcl-2. Celecoxib treatment blocked the phosphorylation and activation of the antiapoptotic kinase, Akt.

Treatment of PC-3 tumor–bearing mice with NS-398 significantly suppressed tumor cell growth and induced regression of existing tumors. Immunohistochemical analysis revealed that the COX-2 inhibitor had no effect on proliferation (proliferating cell nuclear antigen staining), but induced apoptosis (TdT-mediated dUTP digoxigenin nick end labeling [TUNEL]) and decreased tumor microvessel density. The antiangiogenic effect of the compound may be due to an observed decrease in tumor VEGF levels.[43]

One of the most potent in vivo stimulators of VEGF is hypoxia. Studies in colon cancer and vascular endothelial cells have demonstrated a link between COX-2, PGE_2, and hypoxic upregulation of VEGF.[14–16] We tested the effects of cobalt chloride–simulated hypoxia on COX-2 expression in LNCaP, PC-3, and PC-3 ML (an invasive and metastatic subline of PC-3) cell lines. Although all 3 cell lines had some basal COX-2 expression, only the highly invasive and metastatic PC-3 ML subline had the ability to dramatically upregulate VEGF expression (5.6 fold) in response to cobalt chloride treatment. The upregulation of VEGF expression in these cells was accompanied by an upregulation of COX-2 expression (5 fold). Concomitant treatment of PC-3 ML cells with cobalt chloride and a COX-2 inhibitor (NS-398) abrogated the effect of cobalt chloride on VEGF upregulation. Finally, the addition of PGE_2 restored the ability of cobalt chloride to induce VEGF expression, even in the presence of NS-398.[45] These data demonstrate that COX-2 and its product, PGE_2, are involved in hypoxic upregulation of VEGF in prostate cancer cells. The observed effects of NS-398 on prostate tumors in vivo (decreased tumor angiogenesis and VEGF expression)[43] are most likely due to prevention of hypoxic upregulation of VEGF by the COX-2 inhibitor.

SUMMARY AND CONCLUSIONS

There is ample evidence that COX-2 and its PG products are elevated in many human cancers and play a role in various stages of carcinogenesis (Figure 2). The expression of COX-2 in the surrounding stroma may also be important in the paracrine regulation of tumor development. Upregulation of COX-2 may promote tumor development by a va-
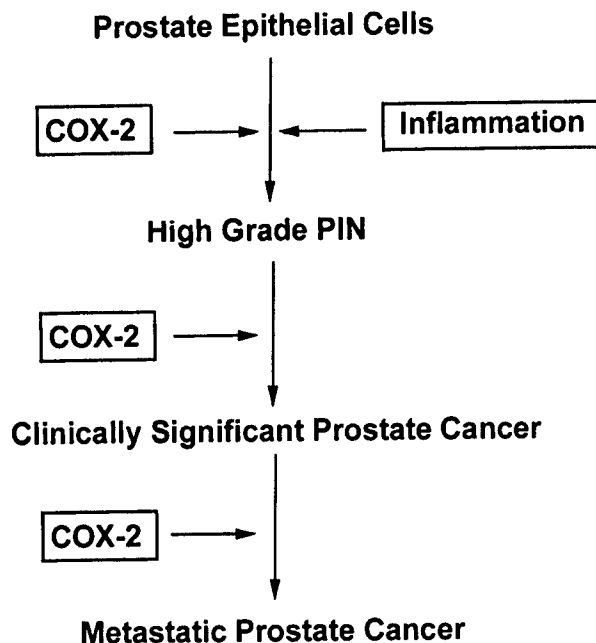
Prostate Epithelial Cells

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┌────────┐                    ┌──────────────┐
│ COX-2  │ ──────▶  ◀──────── │ Inflammation │
└────────┘                    └──────────────┘
                   │
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High Grade PIN

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┌────────┐
│ COX-2  │ ──────▶
└────────┘
             │
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Clinically Significant Prostate Cancer

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┌────────┐
│ COX-2  │ ──────▶
└────────┘
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Metastatic Prostate Cancer

FIGURE 2. *Possible involvement of cyclooxygenase-2 (COX-2) in various stages of prostate carcinogenesis.*

riety of mechanisms, including (1) generation of free radicals and carcinogens; (2) increase in cell proliferation; (3) decrease in apoptosis; and (4) induction of angiogenesis. Selective COX-2 inhibitors may have a role in the chemoprevention and treatment of prostate cancer.

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